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1 Granzyme B is an essential mediator in CD8+ T cell killing of *Theileria*
2 *parva*-infected cells.

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22 Abstract

23 There is established evidence that cytotoxic CD8+ T cells are important mediators of
24 immunity against the bovine intracellular protozoan parasite *T. parva*. However, the
25 mechanism by which the specific CD8+ T cells kill parasitized cells is not
26 understood. Although the predominant pathway used by human and murine CD8+ T
27 cells to kill pathogen-infected cells is granule exocytosis, involving release of perforin
28 and granzyme B, there is to date a lack of published information on the biological
29 activities of bovine granzyme B. The present study set out to define the functional
30 activities of bovine granzyme B and determine its role in mediating killing of *T. parva*-
31 parasitized cells. DNA constructs encoding functional and non-functional forms of
32 bovine granzyme B were produced and the proteins expressed in Cos-7 cells were
33 used to establish an enzymatic assay to detect and quantify expression of functional
34 granzyme B protein. Using this assay, the levels of killing of different *T. parva*-
35 specific CD8+ T cell clones were found to be significantly correlated with levels of
36 granzyme B protein, but not mRNA transcript, expression. Experiments using
37 inhibitors specific for perforin and granzyme B confirmed that CD8+ T cell killing of
38 parasitized cells is dependent on granule exocytosis and specifically granzyme B.
39 Further studies showed that granzyme B-mediated death of parasitized cells is
40 independent of caspases and that granzyme B activates the pro-apoptotic molecule
41 Bid.

43 Keywords

44 Granzyme B, CD8+ T cell, cattle, cytotoxicity, *Theileria parva*, substrate specificity

45

46

47 **Introduction**

48 Antigen-specific CD8⁺ T cell responses have been shown to play a key role in
49 immunity to a number of viral, bacterial and parasitic infections. One such parasite is
50 the tick-borne protozoan *Theileria parva*. *T. parva* infects and transforms bovine
51 lymphocytes resulting in an acute, often fatal, lymphoproliferative disease, which is a
52 major constraint to cattle production in a large part of eastern and southern Africa
53 (1). Following invasion of host lymphocytes, the parasite enters the cytosol where it
54 develops to the schizont stage, which triggers a number of signalling pathways that
55 promote host cell proliferation and inhibit apoptosis. By associating with the mitotic
56 spindle of the activated lymphocyte, the parasite is able to divide at the same time as
57 the host cell, ensuring that infection is retained in both daughter cells. Hence, the
58 parasite remains in an intracellular location during this stage of development. Cattle
59 that recover from infection with *T. parva* are solidly immune to subsequent challenge
60 with the same parasite strain but show variable susceptibility to other parasite strains
61 (2). Development of immunity is associated with a potent parasite-specific CD8⁺ T
62 cell response directed against the parasitized lymphoblasts (3, 4), and transfer of
63 purified CD8⁺ T cells from immune to naïve twin calves has been shown to confer
64 immunity to parasite challenge (5). The mechanism by which CD8⁺ T cells mediate
65 protection against *T. parva* is poorly understood. They exhibit strong MHC-restricted
66 cytotoxic activity and secrete IFN γ and TNF α ; however, unlike other intracellular
67 protozoa (6, 7), these cytokines do not appear to have a direct effector role against
68 the parasite (8). Hence, cytotoxicity is considered likely to have an important role in
69 immunity, although direct evidence for this is lacking and at present there is no
70 information on the molecular mediators of cell killing.

71 As an initial step towards investigating development of subunit vaccines, *T. parva*-
72 specific CD8+ T cell lines have been used successfully to identify a number of target
73 antigens, employing high-throughput screens of expressed parasite cDNAs.
74 Although prime-boost immunisation of cattle with recombinant poxviruses expressing
75 some of these antigens was found to generate specific CD8+ T cell responses, the
76 immunised animals exhibited only partial protection against parasite challenge. A
77 striking feature of the CD8+ T cells induced by this immunisation protocol is that they
78 showed poor cytotoxic activity compared to CD8+ T cells generated by immunisation
79 with live parasites, suggesting poor functional differentiation of the T cell response
80 (9). As with similar results derived from other vaccine trials, these findings highlight a
81 paucity of knowledge of the molecular mechanisms that determine the effector
82 function of vaccine-induced CD8+ T-cells. Understanding the mechanisms of killing
83 of *T. parva*-infected cells by bovine CD8 T cells is required to identify relevant
84 molecular markers that can be used to monitor vaccine-induced immune responses
85 and accelerate vaccine development.

86 Killing of target cells by CD8+ T cells is achieved by release of the contents of
87 secretory lysosomes, known as lytic granules, at the immunological synapse formed
88 upon recognition of class I MHC-bound antigenic peptides by the T cell receptor. Cell
89 killing is initiated by perforin, which creates transient pores in the membrane of the
90 target cell, facilitating uptake into the cytosol of a family of serine proteases known
91 as granzymes. Granzymes exhibit different primary substrate specificities and are
92 able to act on various cellular protein substrates to trigger programmed cell death
93 (10). Five granzymes (A, B, K, H and M) have been identified in humans; mice
94 express four of these granzymes (A, B, K and M) and 6 additional granzymes (C, E,
95 D, F, G and N) (11). We have recently shown that cattle express the same 5

196 granzymes described in humans, plus a novel granzyme (designated granzyme O)
197 (12). Granzymes have been classified into three distinct evolutionary groups, based
198 on their primary substrate specificities, namely trypsin-like (granzymes A and K),
199 chymotrypsin-like (granzymes B, H, C, E, M, D, F, G and N) and metase-like
200 (granzyme M) (13). The most extensively studied of these proteases, granzyme B,
201 cleaves aspartic acid residues. *In vitro* studies have demonstrated that granzyme B
202 induces target cell death by two main pathways, one involving direct proteolytic
203 activation of caspases (leading to DNA damage) and the other by triggering outer
204 mitochondrial membrane permeabilisation via cleavage of the pro-apoptotic protein,
205 BH3-interaction domain death agonist (Bid) (14). The relative physiological roles of
206 these activities *in vivo* remain unclear, particularly in view of the potential functional
207 redundancy among the granzymes. Nevertheless, gene knockout mice deficient in
208 granzyme B have been shown to have reduced levels of CD8+ T cell-mediated
209 cytotoxicity and have increased susceptibility to some viral infections. Despite the
210 residual ability of CD8+ T cells from granzyme B^{-/-} mice to kill target cells, they were
211 unable to induce DNA fragmentation (15). Extrapolation of findings in mice to other
212 mammalian species is also complicated by the finding of differences in protein
213 substrate specificity between murine and human granzyme B; in contrast to human
214 granzyme B, mouse granzyme B is inefficient at cleaving Bid and is therefore
215 believed to rely largely on direct activation of caspases (16).

216 In view of the potential importance of CD8+ T cell mediated cytotoxicity as an
217 effector mechanism against *T. parva*, the current study set out to examine the
218 biological activity of bovine granzyme B and to investigate its role in CD8+ T cell-
219 mediated killing of *T. parva*-infected cells. The results demonstrate that granzyme B

120 plays a key role in killing of parasitized cells, that it is able to cleave Bid and that
121 killing occurs predominantly by a caspase-independent pathway.

122

123 **Results**

124 **Establishing an *in vitro* assay of granzyme B activity.** In order to assess the role
125 of granzyme B in killing of *T. parva*-infected cells, it was necessary to develop
126 methods for measuring its biological activity. Bovine granzyme B expressed in Cos-7
127 cells using the pFLAG eukaryotic expression vector (Figure 1A) was tested for
128 enzymatic activity using a substrate assay employing AC-IEPD-pNA, which contains
129 a tetrapeptide recognized specifically by human and murine granzyme B. As shown
130 in Figure 1B, the active form of Granzyme B (pFLAG-Function - with the pro-
131 dipeptide removed) displayed strong activity against the substrate, whereas the
132 native form (pFLAG-WT) and a version containing a mutation in the active tri-peptide
133 site (pFLAG-Mutant) were inactive. As a substrate-specific control, the chymotrypsin
134 substrate Suc-GGF-pNA was used in the assay and no signal was detected with any
135 of the cattle granzyme B constructs.

136 To confirm the specificity of the expressed granzyme B, the enzymatic activity was
137 measured in the presence or absence of the granzyme B inhibitor AC-IEPD-CHO.
138 The specific inhibitor dramatically reduced the activity of the cattle granzyme B
139 preparation by about 4-fold, close to the background level (Figure 1C), indicating
140 effective inhibitory capacity of AC-IEPD-CHO for cattle granzyme B.

141 **Relationship of cytotoxic activity and granzyme B transcript profiles.** Analysis
142 of cDNA from *T. parva*-specific CD8+ T cell lines by PCR employing primers that
143 amplify transcripts for 6 defined bovine granzymes demonstrated expression of all

144 six genes, including granzyme B (Figure 2A). The kinetics of granzyme B mRNA
145 expression were examined using a semi-quantitative PCR to determine whether
146 expression was strongly influenced by the time interval after antigenic stimulation.
147 Examination of cDNA prepared from CD8⁺ T cells at 2-3 day intervals, between 2
148 and 14 days after stimulation with γ -irradiated *T. parva*-infected cells, demonstrated
149 that near maximal levels of gene expression were achieved between 5 and 7 days
150 after antigenic stimulation, with a subsequent decline in expression (Figure 2B and
151 C). Cells harvested 6-7 days after antigenic stimulation were used for subsequent
152 experiments. To determine whether the levels of killing by CD8⁺ T cells are related
153 to granzyme B and perforin mRNA expression, CD8⁺ T cell clones exhibiting
154 different levels of killing were analysed using a semi-quantitative PCR. Two sets of
155 cloned CD8⁺ T cell lines derived from different animals (641 and 011) were
156 examined; each set of lines expressed identical TCR β chains and recognised the
157 same epitope but exhibited different levels of cytotoxic activity (ranging from 0% to
158 75%) on autologous parasitized cells (Figure 2D). Transcripts for granzyme B and
159 perforin were detected in all 8 T cell clones (Figure 2E). Overall, there was no
160 consistent pattern of either granzyme B ($r = 0.438$, $p = 0.278$) or perforin ($r = -0.104$,
161 $p = 0.806$) mRNA transcript expression that correlated with killing activity (Figure 2F).

162 **Relationship of cytotoxic activity and level of granzyme B protein expression.**

163 A series of CD8⁺ T cell clones specific for the same epitope in the Tp1 *T. parva*
164 antigen (Tp1₂₁₄₋₂₂₄) were used to examine the relationship between killing activity
165 and granzyme B protein expression. These CD8⁺ clones exhibited maximal levels of
166 killing of infected target cells, ranging from 1% to 47%, at effector to target ratios of
167 1:1 or greater (Supplementary 1). Assays of granzyme B were conducted at a
168 standard effector to target ratio of 2:1 to ensure maximal killing activity (Figure 3A).

169 Granzyme B activity in culture supernatants and in cell lysates of these clones
170 following incubation with infected cells was measured using the *in vitro* substrate-
171 specific assay established above. As shown in Figure 3A, the T cell clones showed
172 variable levels of granzyme B release following exposure to antigen-expressing cells
173 (which prior assays had confirmed do not express granzyme B protein, data not
174 shown). The levels of granzyme activity in cell supernatants showed a highly
175 significant correlation with the levels of granzyme protein in lysates of the respective
176 clones ($r = 0.953$, $p < 0.0001$ – Figure 3B), indicating that the levels of enzyme
177 release reflect the cell content rather than inherent differences in rates of release
178 during degranulation. The levels of granzyme B content of the clones also showed a
179 statistically significant correlation ($r = 0.732$, $p = 0.007$) with the levels of cytotoxicity of
180 the T cell clones (Figure 3C).

181 **Cytotoxic activity of T cells is dependent on perforin and granzyme B.** The
182 involvement of lytic granule exocytosis and specifically the role of granzyme B in cell
183 killing by bovine CD8⁺ T cells were investigated by testing the effect of specific
184 inhibitors of perforin and granzyme B. Cytotoxicity assays were first conducted in
185 the presence of a range of concentrations of concanamycin A (CMA), an inhibitor of
186 vacuolar type H⁺-ATPase (17), which raises the pH of the lytic granule and thus
187 induces degradation of perforin (18). The effect of CMA on cytotoxic activity was
188 examined using an un-cloned CD8⁺ T cell line assayed either on *T. parva*-infected or
189 peptide-pulsed target cells, and 3 cloned CD8⁺ T cell lines assayed on infected
190 target cells. Concentrations of 10ng/ml or greater of CMA were found to completely
191 ablate killing of all T cell lines but did not affect the viability of the target cells (Figure
192 4A and B) or the CD8⁺ T cells (data not shown). The results indicate that lysis of *T.*

193 *parva*-infected cells by CD8+ T cells is dependent on perforin, implying that killing is
194 mediated by release of granule enzymes.

195 To examine the role of granzyme B in cell killing, several specific inhibitors used in
196 studies of murine and human CD8+ T cells were first tested for their ability to inhibit
197 granzyme B activity in bovine CD8+ T cell lysates tested using the *in vitro* substrate-
198 specific assay. Although AC-IEPD-CHO was the most potent inhibitor, reducing
199 granzyme B activity by approximately 80% (Supplementary 2), its lack of membrane-
200 permeability, prohibits its use in cellular assays. The membrane-permeable agent Z-
201 IETD-FMK, which inhibits killing by human CD8+ T cells (19, 20), inhibited bovine
202 granzyme B activity by approximately 50% in the substrate assay (Supplementary 2),
203 so was used in subsequent experiments. Pre-incubation of *T. parva*-specific CD8+ T
204 cells with Z-IETD-FMK for one hour prior to use in a cytotoxicity assay resulted in
205 complete inhibition of cytotoxic activity of all 3 cloned T cell lines tested (Figure 4C).
206 A control compound Z-VAD-FMK (a caspase inhibitor that does not affect granzyme
207 B activity of effector cells) did not affect cell killing.

208 In conclusion, these findings reveal that Z-IETD-FMK specifically and effectively
209 blocks the activity of cattle granzyme B and inhibits killing of target cells by bovine
210 CD8+ T cells - indicating that granzyme B is an important mediator for killing of *T.*
211 *parva* infected cells.

212 **Cytotoxic activity of T cells is not dependent on caspases, but is associated**
213 **with activation of Bid.** To examine the role of caspases in cell killing, experiments
214 were undertaken to test the ability of the pan-caspase inhibitor Z-VAD-FMK and its
215 control Z-FA-FMK to block killing by two *T. parva*-specific CD8+ T cell clones. In
216 contrast to previous experiments in which this inhibitor was pre-incubated with
217 effector cells (as a negative control), these experiments involved pre-incubation with
218 the target cells. Cytotoxic activity of the CD8+ T cell clones was blocked by inclusion
219 of inhibitors of perforin and granzyme B (CMA and Z-IETD-FMK respectively) but
220 was unaffected by pre-incubation with Z-VAD-FMK (Figure 5A), demonstrating that
221 the granzyme B-dependent killing by these clones was independent of caspase
222 activity. In contrast, Z-VAD-FMK specifically blocked lysis of *Theileria*-infected cells
223 induced by the pro-apoptotic agent cisplatin (Supplementary 3), which is known to
224 mediate cytotoxicity through caspase induction. These results therefore indicate that
225 granzyme B-mediated killing of *Theileria*-infected cells by specific CD8+ T cells is not
226 dependent on caspases.

227 The other known mechanism by which granzyme B induces cell death is through
228 cleavage, and so activation, of the pro-apoptotic molecule Bid. To investigate this we
229 sought to examine the ability of bovine granzyme B to cleave bovine Bid. Wild-type
230 bovine Bid was expressed in *E.coli* BL21 with cDNA incorporated into the pET-15b
231 expression vector, which carries an N-terminal His-Tag sequence. Purified
232 recombinant bovine Bid protein (Figure 5B) was incubated for 2 hours with serially
233 titrated concentrations of the active form of bovine granzyme B (confirmed using the
234 specific substrate assay) and the reaction products were separated by SDS-PAGE
235 (Figure 5C). Bovine recombinant Bid was cleaved by active bovine granzyme B as
236 revealed by the detection of an N-terminus 11kDa fragment of bovine recombinant

237 Bid of the expected size (based on the predicted cleavage site) by an anti-His-Tag
238 antibody in Western Blot (Figure 5D). A reduction in the concentration of bovine
239 granzyme B was associated with a declining ability to cleave bovine recombinant
240 Bid. Additional smaller bands of approximately 5kDa and 8kDa, present in the
241 Commassie blue-stained gels but not detected in the Western blot, may represent
242 additional smaller fragments of Bid. As controls, an inactive form of bovine granzyme
243 B with a serine to alanine substitution at position 195, mock-transfected cells (pFLAG
244 without an insert) and Cos-7 cells alone were analysed; none yielded truncated Bid
245 products, indicating an inability to cleave bovine recombinant Bid. In conclusion,
246 these results demonstrate that bovine granzyme B cleaves Bid, indicating that
247 cytotoxicity may be mediated by activation of Bid.

248

249 Discussion

250 In this study we aimed to examine the role of bovine granzyme B in the cytotoxic
251 function of *T. parva*-specific CD8+ T-cell responses. To achieve this, we established
252 an *in vitro* substrate-specific assay to detect and quantify expression of bovine
253 granzyme B protein, employing recombinant bovine granzyme B expressed in Cos-7
254 cells. Using this assay, we showed that the levels of killing of different *T. parva*-
255 specific CD8+ T cell clones are significantly correlated with levels of granzyme B
256 protein and that killing of infected cells by bovine CD8+ T cells is mediated by the
257 granule exocytosis pathway and critically requires granzyme B for induction of cell
258 death. Furthermore, we provided evidence that granzyme B-mediated death of
259 parasitized cells is independent of caspases, suggesting that instead the cell death

260 may be induced via activation of Bid, which we show is cleaved by bovine granzyme
261 B.

262 Granzyme B was selected for analysis in this study as it has been shown to be the
263 most potent effector molecule utilized by CD8+ T cells to kill infected cells in both
264 humans and mice. Due to the lack of prior information on bovine granzyme B,
265 studies of its biological activity were required to investigate its role in killing of *T.*
266 *parva*-infected cells. Results obtained with recombinant bovine granzyme B
267 expressed in Cos-7 cells demonstrated many similarities to its human and murine
268 orthologues. This included evidence that processing of the translated polypeptide is
269 similar to that described for humans and mice, with deletion of the dipeptide/G a
270 prerequisite for activation of cattle as well as human and murine granzyme B (21,
271 22). Similarly, mutation of Ser₁₉₅, one of the functional triad of residues at the
272 conserved catalytic site (His, Asp and Ser), was demonstrated to ablate enzymatic
273 activity of the active form of bovine granzyme B confirming, that as with the murine
274 and human proteins, this residue is a critical component of the enzyme's active site
275 (23). These similarities extended to the substrate specificities of the human, murine
276 and bovine forms of granzyme B, with recombinant mature bovine granzyme B
277 showing the capacity to cleave AC-IEPD-pNA. This activity forms the basis of a
278 sensitive and reliable *in vitro* method to measure murine and human granzyme B
279 activity (24).

280 By exploiting this cross-species similarity we were able to generate an equivalent
281 assay for cattle and so investigate levels of biologically active bovine granzyme B
282 and its relation to cytotoxic activity of bovine CD8+ T cells specific for *T. parva*-
283 infected cells, overcoming an obstacle posed by the lack of specific antibodies for
284 bovine granzyme B. The demonstration of strong activity against this substrate

285 confirms that cattle granzyme B displays Aspartase activity, which is a characteristic
286 feature of granzyme B, with no other known serine protease in mammals having a
287 preference for cleaving Aspartic acid-containing substrates (25). We also
288 demonstrated that the non-cell-permeable and cell-permeable compounds AC-IEPD-
289 CHO and Z-IETD-FMK respectively, which are known inhibitors of human and rodent
290 granzyme B (19, 26), efficiently inhibit bovine granzyme B, further highlighting the
291 cross-species functional similarities. However, the inability of another two inhibitors
292 of human and murine granzyme B (Z-AAD-CMK and AC-
293 AAVALLPAVLLALLAPIETD-CHO) to block bovine granzyme B (data now shown)
294 emphasises that extrapolating functional parameters based on orthology cannot be
295 assumed for granzymes and must be empirically validated.

296 This also applies to the pathways utilised by granzyme B to mediate killing, which
297 are known to be species-dependent. Mouse granzyme B predominantly functions
298 through the direct activation of caspases to promote apoptosis, whereas human
299 granzyme B acts mainly via a Bid-dependent pathway (16, 27). Work described in
300 this study demonstrates that bovine granzyme B, like its human orthologue, is
301 capable of cleaving Bid protein *in vitro*, thus providing evidence indicating that Bid
302 activation can potentially be utilised by bovine granzyme B for cell death induction.
303 Although activation of caspases was initially thought to be important in granzyme B-
304 mediated cell death, studies by many groups revealed that requirement for caspase
305 activation, even in mice, isn't absolute. For example, an *in vitro* study of mouse
306 CD8⁺ T cells showed that apoptotic nuclear damage induced by granule exocytosis
307 was abrogated by the caspase inhibitor Z-VAD-FMK, whereas lysis of the cells was
308 unaffected. In contrast, target cell lysis induced by the pro-apoptotic drug cisplatin
309 was specifically blocked by this inhibitor (28). Similar results have been obtained in

310 studies with purified human granzyme B; caspase inhibition preventing granzyme-
311 induced DNA damage but not cell lysis (29). These observations are consistent with
312 the results obtained in this study, which showed that Z-VAD-FMK inhibited cisplatin-
313 induced apoptosis of *Theileria*-infected cells, but did not inhibit granzyme B-mediated
314 cytolytic activity of cattle CD8⁺ T cells.

315 *T. parva* has been shown to enhance the resistance of infected cells to apoptosis by
316 utilizing *NF-κB* activation to induce the expression of anti-apoptotic proteins such as
317 FLIP (which functions as a catalytically inactive form of caspase-8), X-chromosome-
318 linked inhibitor of apoptosis protein (XIAP) and c-IAP (which block caspase-9 and
319 also downstream executioner caspases 3 and 7) (30). Studies by Guernon and
320 colleagues in 2003 showed that drug-induced parasite death in *Theileria*-infected
321 cells resulted in apoptosis involving activation of caspases 9 and 3 and was inhibited
322 by Z-VAD-FMK (31). These findings confirmed that bovine caspases in non-
323 granzyme B mediated killing are capable of inducing cell death and that Z-VAD-FMK
324 is an effective inhibitor of bovine caspases. The inhibition of killing by *T. parva*-
325 specific CD8⁺ T cell clones by Z-IETD-FMK but not Z-VAD-FMK in the current study
326 demonstrates that T cell-mediated killing of *T. parva*-infected cells is dependent on
327 granzyme B but independent of caspases. Although this may be universally
328 applicable to bovine granzyme B mediated cytotoxicity, it is important to note that as
329 a consequence of the negative regulation of caspases by intracellular inhibitors
330 induced by the *NF-κB* pathway in *T. parva*-infected cells the apparent redundancy of
331 caspases might be a feature of this specific biological context.

332 The prime rationale for conducting this study was to better understand the molecular
333 mechanisms that underlie the functional capacity of *T. parva*-specific CD8⁺ T-cells.
334 The critical role that these cells play in mediating immunological protection against *T.*

335 *parva* (29) has led to considerable efforts to identify CD8 T cell target antigens for
336 use in generating novel subunit vaccines (32, 33). A number of *T. parva* antigens
337 recognised by CD8 T cells from immune cattle have been identified and, although
338 they have proved to be immunogenic when used in prime-boost immunisation
339 protocols, the CD8+ T-cells elicited generally exhibited poor cytotoxicity and were
340 poorly protective upon *in vivo* parasite challenge (9). Understanding the discrepancy
341 between immunogenicity and protective efficacy will be critical to defining 'correlates
342 of protection' that can guide subsequent vaccine development. Ongoing work is
343 applying transcriptomics to address this issue. However, such approaches used in
344 isolation have limitations and need to be supplemented by analyses of the functional
345 activities of the specific T cell responses, including the cytotoxic activity of CD8+ T-
346 cells. By confirming the central role of granzyme B in the cytotoxic function, this
347 study provides the knowledge and tools that can be used to refine and enhance the
348 immunological evaluation of T-cell responses induced in future vaccine trials.

349 Our data, from assays of expressed biologically active granzyme B, revealed a
350 statistically significant correlation between the levels of granzyme B enzymatic
351 activity in cell lysates (and supernatants) of cloned CD8+ T cell lines and levels and
352 killing of *T. parva*-infected cells.. Direct evidence that granzyme B is a dominant
353 effector molecule in CD8+ T-cell mediated killing of these parasitized cells was
354 provided by subsequent analysis showing that the membrane-permeable inhibitor of
355 granzyme B, Z-IETD-FMK, reduced *T. parva*-infected cell lysis by these CD8+ T-
356 cells by 70-100%. The highly significant association of levels of granzyme B with the
357 cytotoxic activity of a series of cloned CD8+ T-cell lines indicates that relatively high
358 granzyme B cell content is usually required to achieve maximal cell killing.
359 Nevertheless, one clone (which was inhibited by the granzyme B inhibitor – clone 2

360 Figure 4c) consistently showed low levels of granzyme B content and release but
361 displayed relatively strong killing (Figure 3A). The strong killing shown by this single
362 clone despite modest granzyme B content likely reflects variation in other factors that
363 influence cytotoxicity. This does not detract from the overall conclusion from the
364 study that, at the polyclonal *T. parva*-specific CD8 + T-cell response, granzyme B is
365 a critical mediator of cytotoxic function. There is evidence from *in vitro* studies in
366 humans and mice that other granzymes, in addition to directly mediating cell death in
367 some situations, can synergistically increase the activity of granzyme B. Examples
368 from the literature include: i) Co-transfection of rat basophilic leukemia (RBL) cells
369 with granzyme A and granzyme B in the presence of perforin resulting in enhanced
370 killing of tumour targets in a synergistic manner (34); ii) human granzyme H
371 augmentation of granzyme B-mediated killing of adenovirus-infected cells (35-37) by
372 neutralizing the viral inhibitor of granzyme B (L4-100K assembly protein) (36, 37)
373 and iii) human granzyme M, in addition to inducing cell death of tumor cells directly
374 (38-40), can hydrolyse PI-9, thereby inactivating its inhibitory effect on granzyme B
375 (41). Thus, although our finding of strong inhibition of killing by a granzyme B
376 inhibitor indicates that in general the roles of other granzymes do not play a
377 prominent role in killing of *T. parva*-infected cells, there are clear mechanisms by
378 which for individual T-cells complementary granzyme activities may contribute to
379 CD8+ T killing of *T. parva*-infected cells. Unfortunately, further investigation of these
380 interactions in cattle is hampered by the current lack of specific antibodies and
381 biological assays to measure other bovine granzyme proteins.

382 In conclusion, work described in this paper developed molecular and biochemical
383 methods for measuring the functional activity of bovine granzyme B, in order to
384 determine its role in killing *T. parva*-infected cells by CD8+ T cells. The results

385 provided evidence that killing of parasitized cells occurs by granule-mediated lysis
386 and is substantially dependent on granzyme B. However, cell killing was shown not
387 to be caspase-dependent, and the finding that Bid is cleaved by granzyme B
388 suggests Bid activation through cleavage is a feasible alternative/parallel killing
389 mechanism. This study represents the first dissection of the effector mechanisms
390 employed in killing of target cells by bovine CD8+ T cells and specifically provides
391 the first evidence that granzyme B plays a key role in killing of *T. parva*-infected cells
392 by specific CD8+ T cells.

393

394 **Materials and Methods**

395 **Animals and T cell lines.** Four Holstein-Friesian animals (011, 592, 641 and 633)
396 homozygous for the A10 or A18 MHC I haplotypes were used for the study. Their
397 MHC types were determined by a combination of serological typing (42) and MHC I
398 allele-specific PCR (43). The animals were aged 18–36 month at the outset of the
399 study and were maintained indoors on rations of hay and concentrate. Cattle were
400 immunized against the Muguga stock of *T. parva* (TpM) by infection with
401 cryopreserved sporozoites and simultaneous administration of a long-acting
402 formulation of oxytetracycline as described previously (2). Animals were challenged
403 with a lethal dose of sporozoites on two occasions at ~18-month intervals following
404 immunization. All animal experiments were completed in accordance with the Animal
405 (Scientific Procedures) Act 1986. *T. parva*-specific CD8+ T cell lines and clones were
406 generated from the immune cattle and maintained as described previously (44).

407 **Standard and semi-quantitative PCR assays.** Total RNA was extracted from *T.*
408 *parva*-specific CD8⁺ T cell lines from immunized cattle using Tri-reagent (Sigma)
409 and cDNA was synthesised using the Reverse Transcription System (Promega) with
410 priming by the Oligo (dT)₁₅ primer, both according to the manufacturer's
411 instructions. The primers for granzymes and perforin and the protocols for standard
412 PCR reactions were as previously described (12). For semi-quantitative PCR, the
413 sequences of primers were as follows: granzyme B: 5'-ACT GGA ATC AGG ATG
414 TCC AGA G-3' (Forward), 5'- TTT GGG TCC CCC ACA CAC AG-3' (Reverse) and
415 Gapdh: 5'-ACC CCT TCA TTG ACC TTC AC-3' (Forward); 5'-TTC ACG CCC ATC
416 ACA AAC ATG-3' (Reverse). The PCR reactions were composed of 20pmol of
417 granzyme B/perforin primers and 10pmol of Gapdh primers, 2.5 units BIOTAQ (5
418 units/ul, Bioline), 2.5ul SM-0005 buffer, 0.05ug of cDNA template and nuclease-free
419 water to give a final volume of 25ul. The primers for perforin and the protocol for
420 PCR programme were as described above. Semi-quantified PCR products were
421 analysed by 1.5% agarose gel electrophoresis and the density of the specific bands
422 was measured by computer software (KODAK 1D 3.6 version).

423 **Cloning of bovine granzyme B cDNA constructs.** Full-length bovine wild-type
424 (WT) granzyme B was amplified from cDNA by high fidelity PCR, using primers
425 flanking the coding sequence as previously described (12). The high fidelity PCR
426 protocol was composed of 10pmol of primers, 1.2 unit *Pfu* DNA polymerase (3
427 units/ul, Promega), 10x Buffer with MgSO₄ (Promega), 10mM dNTP, 0.5ug of cDNA
428 template and nuclease-free water to give a final volume of 50ul. The programme
429 used was as follows: 95°C for 2 min, 30 cycles of 95°C for 1min followed by 55°C for
430 0.5 min and 72°C for 2.5 min, and a final extension period of 72°C for 5 min. To
431 generate cDNA encoding active granzyme B, six nucleotides encoding a dipeptide

432 segment in the wild-type granzyme B cDNA (which inhibits granzyme B function and
433 is present in pro-granzyme B but absent in fully mature granzyme B) were deleted by
434 PCR splice overlap extension (PCR-SOE), based on procedures described for
435 human granzyme B (21). Briefly, two PCR assays were initially performed to
436 generate two overlapping fragments that carry the six-nucleotide deletion in the
437 overlapping segment. These reactions utilised the external flanking primers
438 described above with the following internal primers: 5'-
439 CAAAGGCAATCATCGGGGGCCATG-3' (Forward); 5'-
440 CCCGATGATTGCCTTTGCCCTGGG-3' (Reverse). The resulting two fragments
441 were mixed, denatured and annealed to produce deletion mutant DNA templates and
442 amplification of the extended DNAs was performed with flanking primers in a further
443 PCR. Substitution of Ser with Ala at the active site of dipeptide-knockout cDNA was
444 performed by 'megaprimer' PCR mutagenesis (45, 46) using an internal mutagenic
445 forward primer incorporating the mutation as follows: 5'-
446 AGAAAGCTTCCTTTCAGGGGGACGCGG-3'. Briefly, an initial 5 cycles of a PCR
447 reaction containing 50pmol of internal mutagenic forward primer and 2.5pmol of a
448 flanking reverse primer (as described above) was followed by a prolonged extension
449 step to generate mutant mega fragments. 50pmol of the other flanking primer (as
450 described above) was added to the mutant templates and the PCR reaction
451 subjected to a further 25 cycles to generate full-length product containing the
452 mutation. All three bovine granzyme B cDNAs were sub-cloned into the pFLAG-
453 CMVtm-5a expression vector (Sigma) and nucleotide sequencing performed by DBS
454 Genomic (Durham University).

455 **Expression of granzyme B in Cos-7 cells.** Cos-7 cells were maintained in
456 Dulbecco's Minimal Essential Medium (DMEM, Invitrogen) supplemented with 10%

457 FCS, 5×10^{-5} M 2-Mercaptoethanol, 4mM glutamine, 100U/ml penicillin and 100ug/ml
458 streptomycin, at 37°C with 5% CO₂. Cos-7 cells were transfected in 75cm² flasks
459 with the pFLAG-CMVtm-5a vector containing each of the three cattle granzyme B
460 recombinant cDNAs (wild type (60ug), dipeptide knockout (60ug) and knockout with
461 a Ser195Ala substitution (40ug)) or vector only (60ug). The transient transfection
462 was performed by using the Lipofectaminetm 2000 reagent (Invitrogen) according to
463 the manufacturer's protocol. Transfected cells were harvested after 48h, washed and
464 suspended in cold PBS and analysed in further experiments. To test for expression
465 of transfected DNA products, cytospin smears of cells were examined
466 microscopically with anti-FLAG M2 antibody (1:500 dilution; IgG1; Sigma). The
467 transfection efficiency of pFLAG vectors containing three bovine granzyme B
468 recombinant cDNAs containing WT, the dipeptide knockout and the knockout with an
469 additional Ser195Ala substitution was 35%, 33% and 33%, respectively.

470 **Granzyme B protease activity in transfected Cos-7 cells.** Cell lysis and assay of
471 protease activity were performed as previously described for equine granzyme B
472 (47). Briefly, aliquots of 1ml of PBS-washed Cos-7 cells adjusted to 2×10^6 cells/ml in
473 PBS were pelleted and lysed by addition of 0.2ml lysis buffer (1% Triton X-100,
474 50mM Tris, pH8.0 and 2ul of Benzonase Nuclease 25U/ml, Purity>99%, Merck).
475 Following incubation on ice for 20min, lysed cells were centrifuged at 21,000 x g for
476 10min at 0°C to pellet cell nuclei and other cell debris. Supernatants were harvested
477 and assayed in duplicate for protease activity; aliquots of 25ul of lysis supernatant,
478 granzyme B substrate Ac-IEPD-pNA, (Calbiochem) at a final concentration of 300uM
479 and reaction buffer (0.1M Hepes, pH 7.0; 0.3M NaCl; 1mM EDTA) in a total volume
480 of 250ul/well were added into the wells of FalconTM 96-Well Flat bottomed
481 Microplates (BD). The chymotrypsin substrate I, Suc-GGF-pNA (Calbiochem), was

482 used as a negative control for substrate specificity. The reaction was composed of
483 25ul of lysis supernatant, 1mM Suc-GGF-pNA in the final concentration and the
484 reaction buffer (50mM Tris, 100mM NaCl, pH8.0) in a total volume of 125ul. Mixtures
485 were incubated at 37°C for 4h and the colour reaction generated by cleavage of the
486 pNA substrate measured at a wavelength of 405nm by using a Synergy™ HT Multi-
487 Mode Microplate Reader (BioTek). For inhibition of active bovine granzyme B
488 protease activity in lysates, aliquots of 25ul of lysis supernatant containing active
489 bovine granzyme B were pre-incubated with 10uM Ac-IEPD-CHO (the granzyme B
490 inhibitor, Calbiochem) at 37°C for 0.5h.

491 **Granzyme B activity in CD8+ T cell lines.** Methods used for measurement of
492 granzyme B in T cell lysates and supernatants were based on procedures previously
493 described for human and equine granzyme B (24, 47). CD8+ T cells washed in PBS
494 were adjusted to 1×10^6 cells/ml in PBS, pelleted and lysed by addition of 50ul of a
495 lysis buffer per ml as described above. To examine granzyme B release, aliquots of
496 1×10^6 CD8+ T cells were distributed into the wells of 96-well V-bottomed plates
497 together with 5×10^5 target cells in a total volume of 200ul phenol-red-free complete
498 media (RPMI 1640 with 5% FCS, Invitrogen,). Control wells containing effector cells
499 and medium were also included. After incubation in an atmosphere of 5% CO₂ at
500 37°C for 4h, the plates were centrifuging for 10min at 400xg and supernatants were
501 collected. Granzyme B activity was measured by adding aliquots of 10ul of cell
502 lysates or 40ul of culture supernatants in duplicate to wells of Falcon™ 96-well flat-
503 bottomed Microplates (BD) together with 200uM granzyme B substrate, Ac-IEPD-
504 pNA, (Calbiochem) and reaction buffer (0.1M HEPES, pH7.0; 0.3M NaCl; 1mM
505 EDTA) in a total volume of 100ul/well. Wells containing reaction buffer and substrate
506 control were also included as controls. Mixtures were incubated at 37°C for 4h and

507 the colour reaction generated by cleavage of the pNA (p-nitroaniline) substrate
508 measured at a wavelength of 405nm using a Synergy™ HT Multi-Mode Microplate
509 Reader (BioTek). To test for specificity of the reaction, CD8+ T cells were pre-
510 incubated with the cell-permeable granzyme B inhibitor, Z-IETD-FMK (40uM) for 1h
511 prior to preparation and testing of cell lysates as describe above. 40uM Z-VAD-FMK,
512 a pan-caspase inhibitor was used as a negative control, whereas a non-cell-
513 permeable granzyme B inhibitor, AC-IEPD-CHO (10uM) was used to inhibit
514 granzyme B activity in lysates as a positive control.

515 **Cytotoxicity assays.** Standard 4-hour [¹¹¹In]-release cytotoxicity assays were used
516 to measure cytotoxicity of CD8+ T cell clones, using as target cells either autologous
517 *T. parva*-infected cells or autologous *T. annulata* - transformed cells incubated with
518 peptide for 0.5h prior to the assay (44). Peptides were supplied by Pepscan Systems
519 (Lelystad, The Netherlands). All assays were conducted in duplicate, and controls
520 included *T. annulata*-infected target cells without added peptide and, where
521 appropriate, MHC-mismatched *T. parva*-infected target cells. Cytotoxicity assays
522 were established and specific lysis was measured as described previously (44). For
523 inhibition of perforin activity, effector cells were pre-incubated with ten-fold dilutions
524 of concanamycin A (CMA) at final concentrations ranging from 0.1ug/ml to
525 1000ug/ml for 2h at 37 °C. For inhibition of granzyme B activity, effector cells were
526 pre-incubated for 1h at 37°C with 40uM Z-IETD-FMK and the negative control, pan-
527 caspase inhibitor Z-VAD-FMK (40uM). For inhibition of caspase activity, ¹¹¹In
528 labelled target cells were pre-incubated with 80uM Z-VAD-FMK and the negative
529 control, cathepsin B Inhibitor Z-FA-FMK (80uM) for 1h at 37°C.

530 **Generation of recombinant bovine Bid.** Wild-type bovine Bid cDNA was amplified
531 using primers flanking the full-length coding region of bovine Bid as follows: 5'-

532 TAGCATATGGATTTGAAGGTTA-3' (Forward); 5'-
533 TGCTGGATCCGAGTGGTCACTCAGTCCAT-3' (Reverse). The amplified PCR
534 products were purified and sub-cloned into the NdeI and BamHI sides of pET-15b
535 vector (Novagen) and nucleotide sequencing performed by DBS Genomic (Durham
536 University). The protocols for expression and purification of recombinant bovine Bid
537 proteins were performed as previously described for human Bid (48). Briefly, pET-
538 15b expression vectors containing wild-type bovine Bid cDNA were transformed in
539 *E.coli* BL21 (DE3) pLYsS (Novagen) and expressed in the presence of IPTG. The
540 expressed products, which carry an N-terminal His-Tag sequence, were purified with
541 automated immobilised metal affinity chromatography (IMAC) using a nickel affinity
542 column (Qiagen) and further purified with automated ion exchange chromatography
543 (IEC) using a Mono Q column (Pharmacia).

544 **Proteolysis of recombinant bovine Bid by bovine granzyme B.** Two-fold dilutions
545 of lysates containing active bovine granzyme B at final concentrations ranging from
546 10ng to 0.04ng in 10ul reaction volumes were incubated with 3ug of recombinant
547 bovine Bid for 2h at 37°C. Inactive mutated bovine granzyme B (an alanine
548 substitution at position 195), mock (pFLAG without an insert) and Cos-7 cells alone
549 were used as negative controls for granzyme B proteolysis specificity. Reaction
550 products were separated by SDS-PAGE (NuPAGE 4-12% Bis-Tris gel, Thermo
551 Fisher) and visualized by Coomassie blue staining. The reaction products were
552 transferred using the iBlot (Thermo Fisher) for Western blotting, according to the
553 manufacturer's instructions. The blots were probed with anti-His Tag antibody
554 (1:2500 dilution, Thermo Fisher) and anti-FLAG M2 antibody (1:1000 dilution, Sigma)
555 and detected by chemiluminescence using HRP-labelled rabbit anti-mouse IgG
556 (H+L) secondary antibody (1:5000 dilution, Thermo Fisher).

557 **Statistical analysis.** Statistical analyses were performed using Minitab software
558 (Minitab® 15.1.20.0, Minitab Inc.). The correlation between variables was analysed
559 by Pearson's correlation test. P-values < 0.05 were considered significant.

560

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571

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732 Figure 1. (A). Amino acid sequences from nucleotide sequences of three
733 recombinant forms of bovine granzyme B cDNA, aligned with the reference
734 sequence from the genome database. Granzyme B - CDs - the full length cDNA from
735 bovine genome (corr_ENSBTAG00000010057); Granzyme B - WT - pFLAG-CMVtm-
736 5a vector containing wide type granzyme B; Granzyme B - Function - pFLAG-
737 CMVtm-5a vector containing functional granzyme B; Granzyme B - Mutant - pFLAG-
738 CMVtm-5a vector containing functional granzyme B with Ser₁₉₅ to Ala₁₉₅ mutation.
739 Dot-Identical; Dash-Gap; Leader peptide is highlighted in a red box; Dipeptide/GE is
740 in a yellow box; Ser195Ala is in a black box; FLAG epitope-tag sequence of the
741 pFLAG-CMVtm-5a vector is in blue. (B). Enzymatic activity of different recombinant
742 forms of bovine granzyme B tested on a granzyme B-specific substrate AC-IEPD-
743 pNA (Filled bars) and a control substrate Suc-GGF-pNA (Empty bars): Cos-7 cells
744 were transiently transfected with unmodified granzyme B cDNA (WT), cDNA with the
745 GE dipeptide deleted (Function) or cDNA containing a deletion of the dipeptide and
746 an alanine substitution at position 195 (Mutant). The transfection efficiency of Cos-7
747 cells with three granzyme B constructs was 35%, 33% and 33%, respectively.
748 Lysates of the transfected cells collected after 48 hours were incubated with the
749 substrates for 4 hours. Controls consisted of lysates of cells transfected with pFLAG
750 without an insert (Mock) and buffer (No cells) added to the substrate. Colour reaction
751 generated after 4 hours by cleavage of the pNA substrate were measured at a
752 wavelength of 405nm using a Synergy™ HT Multi-Mode Microplate Reader (BioTek).
753 (C). Inhibition of the functional recombinant cattle granzyme B by preincubating with
754 10uM granzyme B specific inhibitor AC-IEPD-CHO for 0.5h.
755
756

757 Figure 2. (A). PCR products obtained for each of the bovine granule enzymes from
758 an uncloned *T. parva*-specific CD8+ T cell line (641). The sizes of the PCR products
759 obtained were: granzyme A (A) - 838bp; granzyme O (O) - 849bp; granzyme B (B) -
760 818bp; granzyme H (H) - 820bp; granzyme K (K) - 889bp; granzyme M (M) - 833bp;
761 Perforin (PFN) - 1275bp; Negative controls (primers with no added cDNA template)
762 were included in the left of the panel. (B). Agarose gels showing the PCR products
763 for granzyme B (457bp), perforin (1275bp) and the GAPDH control (304bp). Days
764 after antigenic stimulation are shown. (C). Changes in quantity of PCR product
765 (vertical axis) at different times following antigenic stimulation, normalised in relation
766 to that of the GAPDH product obtained from the same sample. (D). Cytotoxic activity
767 of 8 *T. parva*-specific CD8+ T cell clones from two different animals (641 and 1011)
768 assayed on autologous *T. parva*-infected targets. (E). Agarose gels showing the
769 PCR products for granzyme B (457bp), perforin (1275bp) and the GAPDH control
770 (304bp) from 8 *T. parva*-specific CD8+ T cell clones (D). (F). Correlation of killing of
771 *Theileria*-infected target cells by CD8+ T cell clones with levels of mRNA expression
772 of granzyme B ($r = 0.438$, $p = 0.278$) and perforin ($r = -0.104$, $p = 0.806$). Changes in
773 quantity of PCR product (vertical axis) in different T cell clones, normalised in
774 relation to that of the GAPDH product obtained from the same sample. (B, E) A
775 negative control (-), without added template, and a positive control (+), consisting of
776 primers with cDNA template of an uncloned *T. parva*-specific CD8+ T cell line (641)
777 day 7 after 3rd stimulation are included. The density of the all PCR amplicon bands
778 was measured by Kodak 1D software (version 3.6). The correlation between
779 variables was analysed by Pearson's correlation test. P-values < 0.05 were
780 considered significant.

781

782

783 Figure 3. (A). Cytotoxic activity and levels of granzyme B content and release of 12
784 *T. parva*-specific CD8+ T cell clones isolated from two animals (641 and 633) were
785 assayed with autologous *T. parva*-infected cell target cells. A standard effector to
786 target ratio of 2:1 was used. Correlation of granzyme B cellular activity with (B) levels
787 of released granzyme B following antigenic stimulation ($r = 0.953$, $p < 0.0001$) and,
788 (C) levels of killing of *Theileria*-infected target cells by CD8+ T cell clones ($r = 0.732$,
789 $p = 0.007$). The correlation between variables was analysed by Pearson's correlation
790 test. P-values < 0.05 were considered significant.

791

792 Figure 4. Inhibition of the cytotoxic activity of (A) an un-cloned ('bulk') CD8+ T cell
793 line from animal 011 and (B) three CD8+ T cell lines from animal 592 by incubation
794 with the perforin inhibitor concanavalin A (CMA), and (C) three CD8+ T cell lines
795 from animal 641 by incubation with the granzyme B inhibitor Z-IETD-FMK. (A, B)
796 Effectors (1×10^4) were pre-incubated with various concentrations of CMA for 2h and
797 tested in a 4-h cytotoxicity assay with [^{111}In]-labelled autologous TpM target cells and
798 MHC-matched target cells pulsed with Tp₂₄₉₋₅₉ peptide (1000ng/ml). (C) Three
799 cloned CD8+ T cell lines (1×10^4) were pre-incubated for 1 h with 40uM Z-IETD-FMK
800 and a negative control, Z-VAD-FMK. Labelled target cells alone were also incubated
801 with the inhibitors in the assay. A standard effector to target ratio of 2:1 was used.

802

803

804 Figure 5. (A). ^{111}In -labelled peptide-pulsed target cells (MHC-matched target cells,
805 5×10^3 + Tp1₂₁₄₋₂₂₄, 100ng/ml) were pre-incubated with the 'pan-caspase' inhibitor Z-
806 VAD-FMK (80uM) for 1h and tested in a 4-hour cytotoxicity assay with two Tp1-
807 specific cloned CD8+ T cell lines from animal 641. As controls, effector cells (1×10^4)
808 pre-incubated with the 'perforin' inhibitor CMA (10ng/ml) for 2h or the 'granzyme B'
809 inhibitor Z-IETD-FMK (40uM) for 1h were tested in the same experiment. Labelled
810 target cell alone were also incubated with these inhibitors in the assay. A standard
811 effector to target ratio of 2:1 was used. (B). Expression vector pET-15b, carrying an
812 N-terminal His-Tag sequence followed by full-length coding sequence of bovine Bid
813 was expressed in *E. coli* BL21 (DE3) in the presence (+) or absence (-) of IPTG and
814 the expressed products were purified using automated immobilised metal affinity
815 chromatography (IMAC) and automated ion exchange chromatography (IEC).
816 Products were separated by SDS-PAGE and visualized by Commassie blue staining.
817 The predicted size of bovine recombinant Bid is 23.7 kD. (C, D) Purified recombinant
818 bovine Bid proteins (3ug) were incubated with indicated concentrations of active
819 bovine granzyme B for 2 h at 37°C. The reaction products were separated by SDS-
820 PAGE and visualized by Commassie blue staining (C) and full-length recombinant
821 Bid and truncated Bid (N-terminus) were detected by anti-His-Tag antibody and
822 recombinant granzyme B was detected by anti-FLAG M2 antibody in Western Blot
823 (D). (C, D) Inactive bovine granzyme B mutant (an alanine substitution at position
824 195), mock (pFLAG without an insert) and Cos-7 cells alone were included as
825 negative controls for granzyme B proteolysis specificity
826









